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Combined CpG and poly I:C stimulation of monocytes results in unique signaling activation not observed with the individual ligands

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ABSTRACT

Toll-like receptors (TLRs) bind to components of microbes, activate cellular signal transduction pathways and stimulate innate immune responses. Previously, we have shown in chicken monocytes that the combination of CpG, the ligand for TLR21 (the chicken equivalent of TLR9), and poly I:C, the ligand for TLR3, results in a synergistic immune response. In order to further characterize this synergy, kinome analysis was performed on chicken monocytes stimulated with either unmethylated CpG oligodeoxynucleotides (CpG) and polyinosinic–polycytidylic acid (poly I:C) individually or in combination for either 1 h or 4 h. The analysis was carried out using chicken species-specific peptide arrays to study the kinase activity induced by the two ligands. The arrays are comprised of kinase target sequences immobilized on an array surface. Active kinases phosphorylate their respective target sequences, and these phosphorylated peptides are then visualized and quantified. A significant number of peptides exhibited altered phosphorylation when CpG and poly I:C were given together, that was not observed when either CpG or poly I:C was given separately. The unique, synergistic TLR agonist affected peptides represent protein members of signaling pathways including calcium signaling pathway, cytokine–cytokine receptor interaction and Endocytosis at the 1 h time point. At the 4 h time point, TLR agonist synergy influenced pathways included Adipocytokine signaling pathway, cell cycle, calcium signaling pathway, NOD-like receptor signaling pathway and RIG-I-like receptor signaling pathway. Using nitric oxide (NO) production as the readout, TLR ligand synergy was also investigated using signaling protein inhibitors. A number of inhibitors were able to inhibit NO response in cells given CpG alone but not in cells given both CpG and poly I:C, as poly I:C alone does not elicit a significant NO response. The unique peptide phosphorylation induced by the combination of CpG and poly I:C and the unique signaling protein requirements for synergy determined by inhibitor assays both show that synergistic signaling is not a simple addition of TLR pathways. A set of secondary pathways activated by the ligand combination are proposed, leading to the activation of cAMP response element-binding protein (CREB), nuclear factor κB (NFκB) and ultimately of inducible nitric oxide synthase (iNOS). Since many microbes can stimulate more than one TLR, this synergistic influence on cellular signaling may be an important consideration for the study of immune response and what we consider to be the canonical TLR signaling pathways.

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1. Introduction

Toll-like receptors (TLRs) are the pattern recognition receptors (PRRs) of the innate immune system that play a crucial role in the first line host defense against microbial pathogens. Each TLR member, either individually or acting with another TLR, recognizes specific pathogen-associated molecular patterns (PAMPs) [1]. Recently, emerging evidence indicates that TLRs cooperate and cross-talk when engaging multiple agonists, and these interactions can result in either suppressing or synergizing a particular immune response. For example, co-stimulation of mouse peritoneal macrophages with the TLR2 and TLR4 ligands, MALP-2 and LPS, markedly increases TNF-α production [2]. Treatments of murine macrophages with a combination of TLR9 and TLR3 agonists synergize to produce nitric oxide (NO), IL-12, TNF-α, and IL-6 [3]. Human TLR3 and TLR4 are also shown to act in synergy with TLR7, TLR8, and TLR9 for the induction of IL-12p40 and IL-12p35 genes in dendritic cells (DCs) [4]. Co-stimulation with unmethylated

Abbreviations: TLRs, Toll-like receptors; CpG, unmethylated CpG oligodeoxynucleotides; poly I:C, polyinosinic–polycytidylic acid; NO, nitric oxide; iNOS, inducible nitric oxide synthase; CREB, cAMP response element-binding protein; NFκB, nuclear factor κB; PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells; GO, Geneontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; STRING, Search Tool for the Retrieval of Interacting Genes; Jak-STAT, Janus kinase–signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; CaMK2, calcium/calmodulin-dependent protein kinase 2; CaM, calmodulin; JNK, Jun N-terminal kinase; PKB, protein kinase B; AKT, RAC-alpha serine/threonine-protein kinase 1/2; AP-1, activator protein 1; TAK1, TGF-beta activated kinase 1; TBK1, TANK-binding kinase 1; PI3K, phosphoinositide 3-kinase; Erk, extracellular signal-regulated kinases; C/EBP, CCAAT/enhancer binding protein; PLCγ, phospholipase C gamma; RAC1, Ras-related C3 botulinum toxin substrate 1; BLNK, B cell linker protein; BTK, Bruton tyrosine kinase; ASK1, apoptosis signal-regulating kinase 1; MKK3, mitogen-activated protein kinase kinase 3; MKK6, mitogen-activated protein kinase kinase 6; MK2, mitogen-activated protein kinase-activated protein kinase 2; PKC, protein kinase C; RAS, Rat sarcoma; RAF, rapidly accelerated fibrosarcoma; RSK1 also referred to as p90RSK, ribosomal S6 kinase 1.

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CpG oligodeoxynucleotides (CpG) and flagellin synergistically enhances the secretion of IL-10 and IFN-γ but conversely inhibits INF-α production in human peripheral blood mononuclear cells (PBMCs), monocytes, and monocyte-derived DCs [5]. TLRs 2 and 3 act in concert to induce inflammatory cytokines TNF-α, IL-6, and IL-12p40 in mouse DCs but down-regulate TLR3-induced expression of IL-12p35 [6]. In human monocyte-derived macrophages and DCs, combinatory stimulations of the TLR8 ligand, together with the TLR3 or TLR4 ligand, led to synergistic expression of IL-6, IL-10, IL-12, and TNF-α mRNA [7].

Chickens are known to express an orthologue of mammalian TLR3 that recognizes polyinosinic–polycytidylic acid (poly I:C), functioning as the receptor for dsRNA [8–10]. However, chickens are unique among vertebrates as an orthologue of the mammalian TLR9 is missing from the chicken genome [11,12]. Instead of TLR9, chicken TLR21, an orthologue to the TLR21 found in fish and amphibians [13], was found to recognize CpG and act as a functional homologue to the mammalian TLR9 [14,15]. Interaction between TLR3 and TLR21 agonists poly I:C and CpG has been reported to synergize in the expression of proinflammatory cytokines and chemokines and the production of NO in chicken monocytes [16,17]. The synergistic interaction between the TLR3 and TLR21 pathways also produces a stronger Th1-biased immune response in chicken monocytes [18].

Peptide arrays for kinome analysis are a high-throughput means of studying kinase activity and cellular signaling. They have been used previously to study topics ranging from cancer biology [19] to host–bacterial ligand interactions [20]. The initial peptide arrays were designed for the study of human and mouse; subsequently, peptide arrays were designed to study other species of research interest, initially bovine and ovine [21]. These species-specific peptide arrays have been used to study signaling involving host–bacteria interactions [22], host–viral interactions [23] and prion biology [24], among others. Our group recently designed and utilized the first reported chicken-specific peptide array, which was also the first kinome peptide array used to study in vivo metabolism [25]. Here, we introduce the first chicken-specific peptide array designed for immunological study. To our knowledge, this is also the first report of the use of high-throughput peptide array kinomics to study TLR ligand synergy.

As described above, numerous reports of the synergistic affects of multiple TLR ligands have been published, but the mechanisms of many of these synergistic responses have not been well characterized. Here, we specifically consider the synergy induced by the TLR ligands CpG and poly I:C by using peptide arrays to look at a broad cross section of cellular signaling. Peptide arrays were combined with the use of selective signaling-protein inhibitors to validate the arrays and identify proteins required for a synergistic response. We show that the synergistic response induced by CpG and poly I:C induces novel cellular signal transduction, and we report on potential mechanisms by which this synergy is being achieved based on the activation of transcription factors cAMP response element-binding protein (CREB) and nuclear factor κB (NFκB).

2. Materials and methods

2.1. Ethics statement

These studies were approved by the Animal Care and Use Committee (ACUC) at the Southern Plains Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture (ACUC #2012007), which meets all federal requirements as defined in the Animal Welfare Act, the Public Health Service Policy, and the Humane Care and Use of Laboratory Animals.

2.2. Reagents

Synthetic oligodeoxynucleotides were purchased from TriLink BioTechnologies (San Diego, CA, USA). The sequence of synthetic CpGs used in the present study was GTC GTT GTC GTT GTC GTT [26]. The synthetic dsRNA analog poly I:C was obtained from InvivoGen (San Diego, CA, USA). Cell culture medium and reagents were obtained from Sigma (St. Louis, MO, USA). The inhibitors PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), (52)-7-Oxozeaenol (TAK1 inhibitor), AKTi1/2 (PKB/AKT1/2 inhibitor), BX795 (TBK1 inhibitor), Bay11-7082 (NFκB inhibitor) and LY294002 (PI3K inhibitor) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The inhibitor SR11302 (AP-1 inhibitor) was obtained from Tocris Bioscience (Bristol, UK). The inhibitors AG490 (Jak1/STAT1,3 inhibitor) and KN-93 (CaMK2 inhibitor) were obtained from Cayman Chemical (Ann Arbor, MI, USA). The inhibitor W-7 (CaM inhibitor) was obtained from Enzo Life Sciences (Plymouth Meeting, PA, USA). The calcium chelator BAPTA-AM (calcium chelator) was obtained from Sigma (St. Louis, MO, USA).

2.3. Cell isolation, monocyte culture, and stimulation

Chicken PBMCs were isolated from peripheral blood collected from 2- or 3-day-old chickens as previously described [16]. Aliquots of 1 ml of PBMC (1×10^7 cells/ml) were dispensed into a 24-well plate and incubated at room temperature (~22 °C) for 2 h. After incubation, non-adherent cells were removed by washing 3× with RPMI-1640. The adherent-enriched monocytes were cultured for 18 h in a complete DMEM medium [Dulbecco's Modified Eagles Medium containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), and 1.5 mM L-glutamine]. Prior to stimulation, cells were washed once more with fresh media. Cells were then stimulated with CpG (5 μg/ml), poly I:C (25 μg/ml), or a combination of the two for 24 h at 41 °C in a 5% CO_2 and 95% humidity incubator. For peptide array samples, cells were stimulated with CpG, poly I:C or the combination for 1 h or 4 h. Cells were then collected and pelleted in a centrifuge at 300 ×g. Supernatant was removed, cell pellet was snap frozen in liquid nitrogen, and the pellet was transferred to a −80 °C freezer. In the calcium chelation studies, cells were pretreated with inhibitors or BAPTA-AM at various concentrations as indicated for 1 h and then stimulated with CpG (5 μg/ml), poly I:C 25 (μg/ml) or the combination for an additional 24 h.

2.4. Peptide arrays

Samples were taken from the −80 °C freezer and lysed using 100 ul of lysis buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 μg/ml leupeptin, 1 g/ml aprotinin and 1 mM Phenylmethylsulphonyl fluoride (all products from Sigma Aldrich (St. Louis, MO), unless indicated)]. The peptide array protocol was carried out as per Jalal et al., 2009 [21] with alterations described in Arsenault et al., 2012 [22]. Data normalization and principal component analysis (PCA) was performed as per Li et al., 2012 [27]. Geneontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by uploading the statistically significant peptide lists to the Search Tool for the Retrieval of Interacting Genes (STRING) [28].

2.5. Nitrite assay

Nitrite, a stable metabolite of nitric oxide produced by activated monocytes, was measured by the Griess assay [29]. Briefly, an aliquot of 100 μl culture supernatant from each well was transferred to the wells of a new 96-well flat-bottom plate and combined with 50 μl of 1% sulfanilamide and 50 μl of 0.1% naphthylenediamine (both prepared in 2.5% phosphoric acid solution). After a 10 min incubation at room temperature, the nitrite concentration was determined by measuring the optical density (OD_{550}) of each well using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in

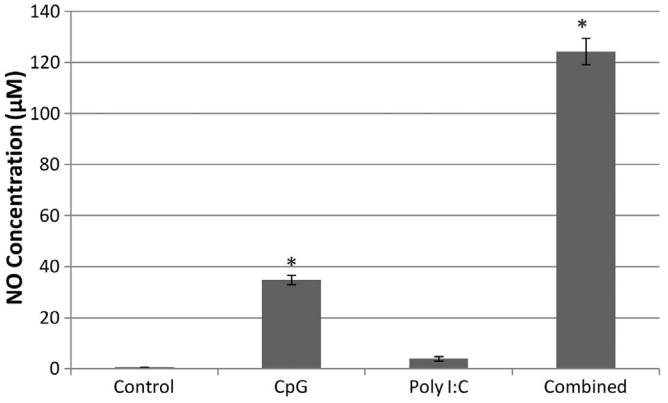

Fig. 1. Heatmap of significant differential peptide phosphorylation. A heatmap generated incorporating the statistically significant ($p < 0.05$) peptide phosphorylation events. Phosphorylation fold change relative to control is indicated by the color, red indicates increased phosphorylation and green decreased phosphorylation.

the cell-free medium. At least three independent experiments were conducted at different times. Within each experiment, nitrite levels from three to five replicate wells of cell culture were measured for each treatment. Data were analyzed by One Way ANOVA, followed by multiple comparisons (Tukey test) using SigmaStat® software (Jandel Scientific, San Rafael, CA). The value of $p < 0.05$ is considered to be significant.

3. Results

3.1. Kinome analysis

At 1 h and 4 h post stimulation, kinome analysis was conducted on monocytes stimulated with CpG alone, poly I:C alone, and a combination of the two. Each stimulated sample was compared to a time-matched control of untreated cells to determine the change in phosphorylation state due to the stimulation. The complete results can be seen in Supplementary Table 1. The statistically significant ($p < 0.05$) phosphorylation events are shown in Fig. 1. A large number of peptides were shown to be influenced by the stimulations.

The kinome analysis of chicken monocytes under the stimulation conditions revealed both commonalities and differences between the synergistic and individual ligand stimulations. The unique peptides were the most interesting, as they underscore the differences between the three stimulation conditions. At the 1 h time point, kinome results of CpG-stimulated cells showed 19 unique peptides differentially

Table 1
Unique peptide phosphorylation.

1 h stimulation			4 h stimulation		
CpG	Poly I:C	CpG + poly I:C	CpG	Poly I:C	CpG + poly I:C
14-3-3beta S183	4E-BP1 T46	ADCY2 S1062	ACC1 S81	ATF2 T72	14-3-3beta S183
CARD9 S238	ACC1 S81	BLNK Y91	ACTA1 S158	EEA1 T1390	Abl2 Y224
CDK2 T160	Casp8 S350	Btk Y549	AKT1 T308	IL4R Y230	Abl2 Y402
CDK2 Y15	CD28 Y192	CCR2 Y130	AKT1 S473	MAPKAPK2 T276	ARMS S2364
FGFR4 Y859	Cdc42 Y64	Cdc25A T510	AKT3 T305	MAPKAPK5 T176	CAMK4 T185
IkB-alpha S36	Cot S400	CREB S119	AMPK1 S173	Met Y1350	Cbl Y728
IRF-7 S464	CTNNB1 T41	CSFR Y813	APP T507	PIK3R2 Y612	CDK2 T160
Jun S69	CSK S364	CTNNB1 S675	A-Raf S343	Rab5b S123	CSFR Y813
MSK1 T571	Daxx S363	ERK1 T193	Btk Y549	SOCS3 Y205	CSK S364
p90RSK S381	EEA1 T1390	ERK3 S189	Caspase6 S268	STAT3 Y706	ERK1 T193
PAK1 T212	eEF2K S366	Ezrin T566	CCR2 Y130	Syk Y503	Ezrin Y477
PDK1 Y379	FRS2 Y196	Fas Y265	CCR5 S276	TrKA Y785	fyn Y417
PIP5K S307	gp130 Y764	FLT3 Y542	CTNNB1 S675	TrkC Y518	gp130 S878
SOS1 S1234	gp130 Y905	HDAC4 S628	Etk 514	VIM S34	IL2RB Y462
STAT6 Y236	IFIH1 S299	HSP70 Y528	FAK Y397		IL2RB Y270
TAK1 S446	Jak2 Y1004	IFNAR1 S547	gp130 Y905		JNK2 T183
TAO1 T784	jak3 Y784	IGF1R Y1166	Grb10 S478		MEKK1 S122
VIM S34	Keap1 Y272	IGF2R S2392	HS1 Y382		MKK3 T133
XIAP S87	MnK1 T239	iNOS Y148	HSP60 Y227		Mnk2 T172
	NFAT2 S302	Jak2 Y219	IFNAR1 Y486		NFAT1 S225
	NFAT2 S351	JIP1 T133	IGF2R S2464		NFE2L2 Y554
	PIK3R1 S608	MDM2 S160	Jun S69		PDGFRb Y745
	PPP2CA T304	Met Y1235	LAB Y118		PDK1 S244
	PTEN T382	p38-alpha Y323	Met Y1235		PKCE S369
	Raf1 S338	p38beta Y181	MSK1 S366		PKD3 S734
	RSK2 Y674	p70S6K T389	p38-alpha Y323		PTEN Y240
	Shc1 Y47	p90RSK S398	p67phox T233		Rab14 Y14
	Smad2 T8	PIK3R2 Y471	PIK3R2 Y471		Raf1 S338
	SOS1 S1208	PKCE S369	PKCD T530		Smad2 S245
	TIRAP Y78	PXN Y118	PTEN S370		SOCS3 Y188
	TRAF2 T117	Rab7 Y183	Pyk2 Y405		Src Y416
	WASP Y256	Shc1 Y376	PXN Y31		TAB1 S421
		Smad3 S209	Shc1 Y376		TAO1 S989
		STAT1 Y673	SOS1 S1234		TBK1 S172
		STAT5B Y740	STAT1 S729		Tyk2 Y35
		STMN1 S16	STAT4 S722		VEGFR-2 Y943
		STMN1 S38	STAT6 Y236		WASP Y256
		tuberin S940	STMN1 S16		WNT Y552
		VASP S243	Syk Y330		
			VEGFR-1 Y1322		
			VEGFR-1 Y786		
			VEGFR-2 Y1207		

Peptides which were uniquely significantly differentially phosphorylated (p-value < 0.05) relative to control at each time point are listed. The peptides listed are those which only appear under one ligand stimulation condition per time point.

phosphorylated relative to control, which did not appear in the other two stimulations. Poly I:C-stimulated cells showed 32 unique differential peptides. Finally, the combined stimulation results showed 39 unique peptides (Table 1). Similar results were observed at the 4 h time point (Table 1), when cells were treated with CpG, 42 unique peptides displayed differential phosphorylation. When cells were stimulated with poly I:C for 4 h, 14 unique peptides were differentially phosphorylated. Cells treated with the combination of ligands displayed 38 uniquely differentially phosphorylated peptides. These results indicated that there were unique responses occurring due to each of the three stimulations that did not occur with the others.

The peptides shown to be significantly differentially phosphorylated by the three stimulations at the two time points were analyzed using the KEGG pathway analysis function of the STRING database [30,31]. Table 2 shows the generated pathway results common to the three stimulation conditions at each time point; for example, the Apoptosis pathway was altered by CpG stimulation, poly I:C stimulation and the combination of CpG and poly I:C at the 1 h time point. Six pathways were altered by all stimulations at both time points. These pathways were B cell receptor signaling pathway, the Janus kinase–signal transducers and activators of transcription (Jak-STAT) signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, Neurotrophin signaling pathway, non-small cell lung cancer and pathways in cancer. Since both ligands were TLR stimulants and are part of the innate immune system, it is understandable that there would be at least some consistent protein phosphorylation and pathway activation between them.

CpG and poly I:C activate distinct TLRs, and the combination of the two ligands results in synergistic immune effects [16–18]. Table 1 shows the unique peptide phosphorylation events that occurred when cells were stimulated with both CpG and poly I:C. These unique events strongly indicated that novel cellular activities were induced by the combination of the two ligands. In order to better understand this synergy, we analyzed the pathway results and looked for pathways that were unique to the combination. Table 3 shows unique pathways that were altered by the combination of CpG and poly I:C at each time point. These were pathways that did not appear following CpG or poly I:C stimulation alone. There were several different pathways which are altered between the 1 h and 4 h time points. Two pathways are highlighted that are altered by the combination of the two ligands at both time points: the calcium signaling pathway and the Aldosterone-regulated sodium reabsorption pathway.

Table 2
Consistent pathways activated by poly I:C, CpG and the combination.

Consistent pathways 1 h	Consistent pathways 4 h
Apoptosis	Acute myeloid leukemia
B cell receptor signaling pathway	**B cell receptor signaling pathway**
Chemokine signaling pathway	Fc epsilon RI signaling pathway
Chronic myeloid leukemia	Fc gamma R-mediated phagocytosis
Endometrial cancer	Glioma
ErbB signaling pathway	VEGF signaling pathway
Focal adhesion	
Jak-STAT signaling pathway	**Jak-STAT signaling pathway**
MAPK signaling pathway	**MAPK signaling pathway**
mTOR signaling pathway	
Neurotrophin signaling pathway	**Neurotrophin signaling pathway**
Non-small cell lung cancer	**Non-small cell lung cancer**
Pathways in cancer	**Pathways in cancer**
Renal cell carcinoma	
T cell receptor signaling pathway	
Toll-like receptor signaling pathway	

Peptides that were significantly differentially phosphorylated relative to control were entered into the STRING database to find pathways within the data. The table shows the pathways that were significantly affected across the three stimulation conditions at each time point. The pathways in bold are those that were significantly altered by the three stimulations across both time points, in effect those that were affected by all stimulations at both time points.

Table 3
Unique pathways activated by CpG and Poly I:C synergy.

Poly I:C + CpG at 1 h	Poly I:C + CpG at 4 h
Aldosterone-regulated sodium reabsorption	Aldosterone-regulated sodium reabsorption
Axon guidance	Adipocytokine signaling pathway
Bladder cancer	Cell cycle
Calcium signaling pathway	Calcium signaling pathway
Cytokine–cytokine receptor interaction	Long-term potentiation
Endocytosis	NOD-like receptor signaling pathway
Gastric acid secretion	RIG-I-like receptor signaling pathway
Long-term depression	
Oocyte meiosis	
Primary immunodeficiency	
Thyroid cancer	

Peptides that were significantly differentially phosphorylated relative to control following the stimulations were entered into the STRING database to find pathways within the data. The results from CpG stimulated alone, poly I:C stimulated alone and the combination were compared. The table shows those pathways that were generated by the combination of CpG and poly I:C but were not found in either CpG alone or poly I:C alone results.

3.2. Nitric oxide response

It had previously been reported that a significant increase in cellular NO release was one of the observed responses to the CpG and poly I:C combination in monocytes [16]. We again showed that synergistic response here; poly I:C stimulation had no significant influence on NO release, CpG stimulation induced significant NO release, and the combination of CpG and poly I:C resulted in a synergistic increase in NO release well above that seen with CpG alone (Fig. 2). Using NO as the readout of the CpG-induced and synergistic TLR response, we analyzed various signaling protein inhibitors to confirm both the peptide array results and the unique pathway activation resulting from the ligand combination. The inhibitors were selected based on their key roles in TLR-related innate immune pathways. Observing whether an inhibitor reduced or eliminated the NO release, induced by either CpG or the ligand combination, indicated whether or not the involved pathway or pathway branch was required for a NO response.

The inhibitor data was in good agreement with the peptide array data and provided additional evidence for unique pathway activation by the ligand combination. The inhibitors tested included those for p38 MAPK, calcium/calmodulin-dependent protein kinase 2 (CaMK2), calmodulin (CaM), Jun N-terminal kinase (JNK), protein kinase B/RAC-alpha serine/threonine-protein kinase 1/2 (PKB/AKT1/2), NFκB, activator protein 1 (AP-1), TGF-beta activated kinase 1 (TAK1), TANK-binding kinase 1 (TBK1), Jak2/STAT1/3, phosphoinositide 3-kinase (PI3K), and extracellular signal-regulated kinases 1/2 (Erk1/2), and all had a significant effect on CpG-induced NO release (Figs. 3–5). Several inhibitors also had a significant effect on the NO release induced by the ligand combination. These included p38 MAPK, CaMK2, CaM, JNK, PKB/AKT1/2, NFκB, PI3K and Erk1/2 (Figs. 3 and 5). The peptide array results showed that p38, CaMK2, JNK, PKB/AKT1/2, AP-1, TAK1, TBK1, Jak2/STAT1, PI3K, and Erk1 were all differentially phosphorylated by CpG and/or the combination of CpG and poly I:C. These results indicated that similar signal transduction proteins were being implicated by both the peptide array results and the inhibitor results in the CpG and CpG poly I:C stimulated cells.

The effects of the inhibitors on NO release fell into three categories: the inhibitor reduced/eliminated CpG-induced NO release but not synergistic NO release; the inhibitor reduced/eliminated both CpG-induced NO release and synergistic NO release; the inhibitor reduced CpG-induced NO release and to a lesser extent synergistic NO release. Inhibitors that reduced/eliminated CpG-induced NO release but not synergistic NO release included inhibitors for AP-1, TAK1, TBK1, Jak2/STAT1,3 (Fig. 4). The results indicated that these signaling proteins were not required to induce a synergistic response and were only part of the pathway activated by CpG alone. The inhibitors

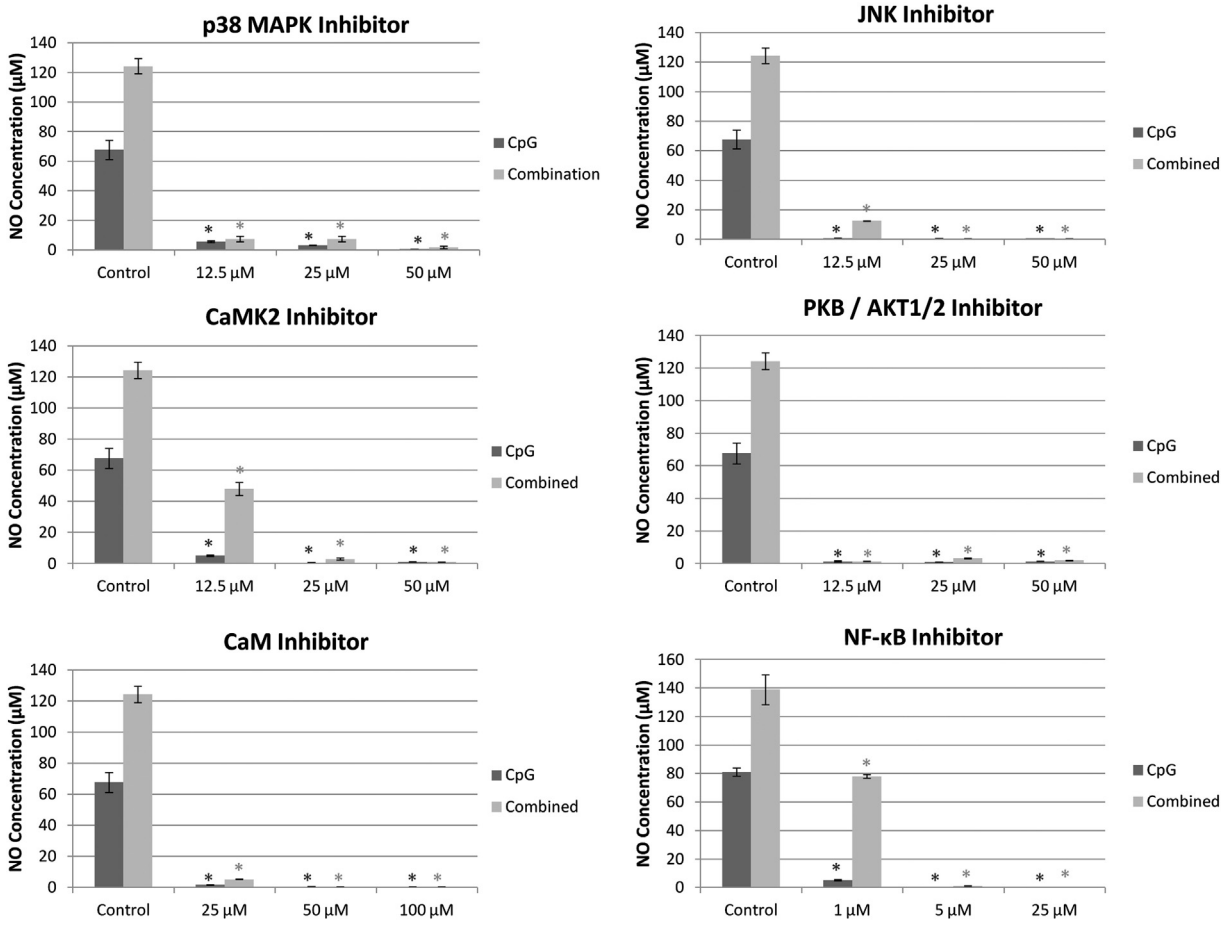

Fig. 2. Ligand-induced NO release. Monocytes were stimulated with CpG, poly I:C or the combination of the two. The concentration of NO released by the stimulations was measured. Results are expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. The * indicates a statistically significant difference ($p < 0.05$) from control.

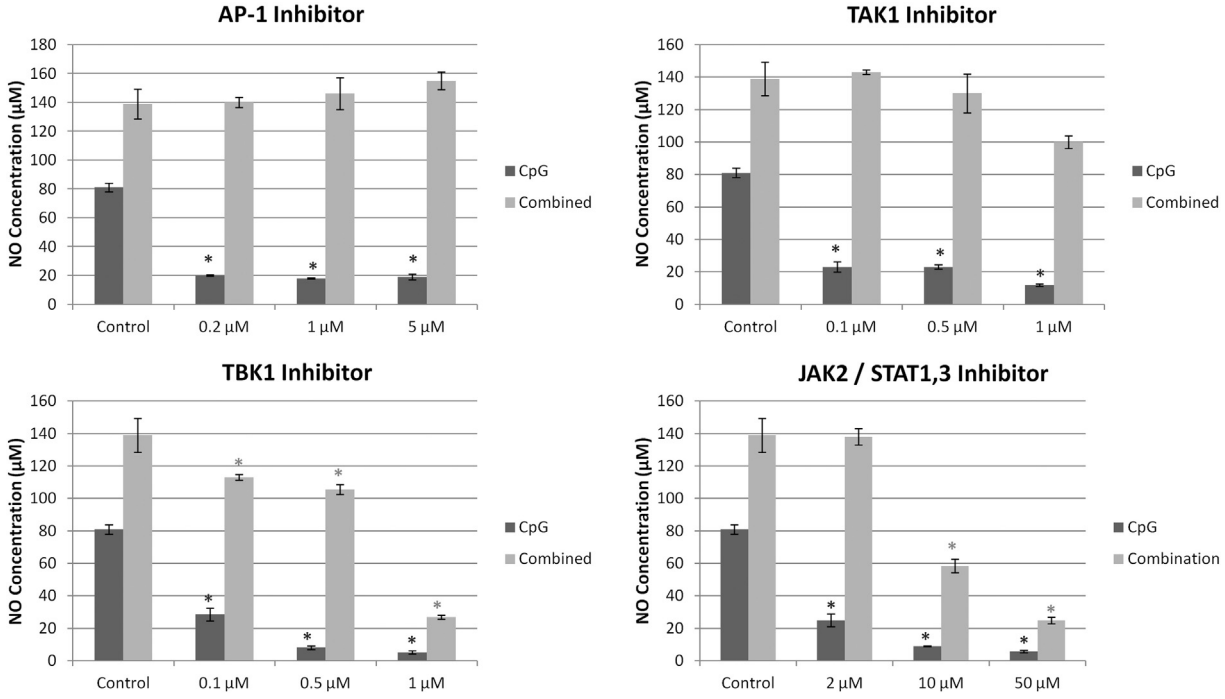

Fig. 3. Signaling proteins required to induce NO release. Inhibitors for each of the indicated signaling proteins were assayed for effect on NO release in cells stimulated with either CpG or the combination of CpG and poly I:C. Results are expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. The * indicates a statistically significant difference ($p < 0.05$) from control.

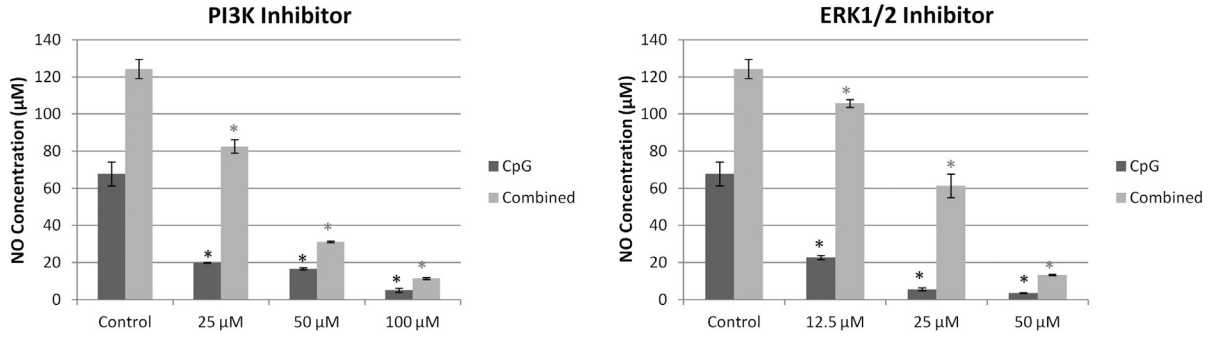

Fig. 4. Signaling proteins required to induce NO release following CpG stimulation but not CpG and poly I:C stimulation. Inhibitors for each of the indicated signaling proteins were assayed for effect on NO release in cells stimulated with either CpG or the combination of CpG and poly I:C. Results are expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. The * indicates a statistically significant difference ($p < 0.05$) from control.

that reduced/eliminated both CpG-induced NO release and synergistic NO release were proteins required for NO release. The proteins that fell into this category were p38, JNK, CaMK2, CaM, PKB/AKT and NFκB (Fig. 3). The proteins that when inhibited reduced CpG-induced NO release and to a lesser extent synergistic NO release included PI3K and ERK1/2 (Fig. 5). This response indicated that following stimulation with CpG alone NO release was dependent on these proteins; while the synergistic response was only partially dependent on their signaling.

The peptide array data, pathway analysis and inhibitor results all indicated that calcium was involved in the NO-release response to the combination of CpG and poly I:C. The inhibitor results also pointed to a role for calcium-related proteins in CpG-induced NO release. In order to confirm calcium's role in CpG and the ligand combination induced NO release we assayed the NO-release response with and without the calcium chelator BAPTA-AM. The chelating of the intracellular calcium by BAPTA-AM eliminated the NO response of the cells when stimulated by CpG and by CpG in combination with poly I:C (Fig. 6). These results indicated a central role for calcium release or calcium-dependent signaling intermediates in the induction of a NO response in these cells.

4. Discussion

The synergistic NO-release response of monocytes stimulated with the CpG and poly I:C combination has been observed previously [16,17]. Although poly I:C did not independently induce a NO response, it was possible that the simple combination of TLR3 and TLR21 signaling caused a greater NO release than was observed with CpG alone. In this study two significant results showed that rather than an additive signaling of the two pathways, the combination of CpG and poly I:C caused unique signaling events that were not observed with either ligand alone. The peptide array data showed a number of unique peptides which displayed phosphorylation following stimulation with the ligand combination. These peptides were unaffected by either CpG or poly I:C stimulation alone (Table 1). The addition of the inhibitors for AP-1, TAK1, TBK1 and Jak2/STAT1,3 resulted in the elimination of CpG-induced NO release, while the combination of CpG and poly I:C still induced a NO release response (Fig. 4). The observed NO release indicates that the combination stimulation was not dependent on AP-1, TAK1, TBK1 or Jak2/STAT1,3 activity, though CpG stimulation required these proteins for NO release. The signaling molecules AP-1, TAK1, TBK1, Jak and STAT are all members of the classical TLR response pathways (KEGG: ko04620). The signaling induced

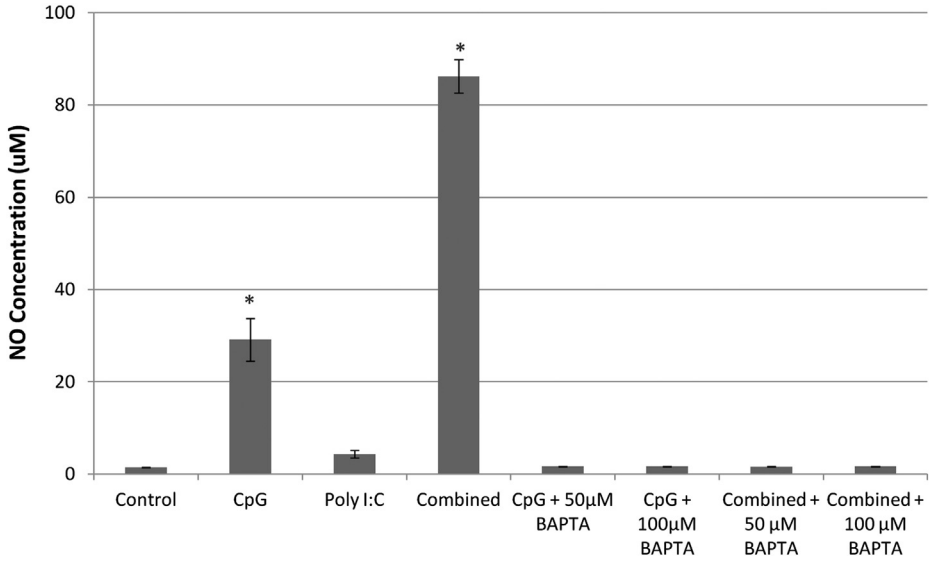

Fig. 5. Signaling proteins for which their inhibition reduced CpG induced NO release and the combination of CPG and poly I:C induced release to a lesser extent. Inhibitors for each of the indicated signaling proteins were assayed for effect on NO release in cells stimulated with either CpG or the combination of CpG and poly I:C. Results are expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. The * indicates a statistically significant difference ($p < 0.05$) from control.

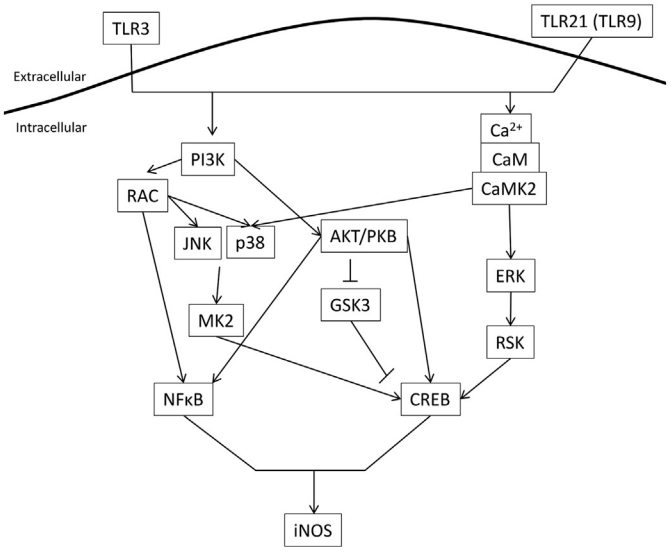

Fig. 6. Effect of calcium chelation on NO release. The effect of calcium chelator BAPTA on CpG and the combination of CpG and poly I:C induced NO release was assayed. Results are expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. The * indicates a statistically significant difference ($p < 0.05$) from control.

by the ligand combination must utilize other signaling molecules and a transcription factor other than AP-1.

The inhibitors of PI3K and ERK1/2 reduced or eliminated CpG induced NO release but have a smaller effect on the NO release induced by the ligand combination. This indicates that, CpG-induced response was dependent of these proteins, whereas the synergistic response utilized these proteins but was not entirely dependent on them to elicit a NO release.

A subset of proteins were universally required for NO release in this study, these were p38 MAPK, JNK, CaMK2, PKB/AKT1/2, NFκB and CaM. With the inhibition of these protein activities, the induction of NO release was not possible with either CpG alone or the ligand combination (Fig. 3). These results indicated that the signaling pathways involved in both the CpG and the combination response must incorporate these proteins.

The results of this study strongly point to a role for calcium and calcium signaling in the synergistic NO response. Table 3 shows that, at both 1 h and 4 h post stimulation, the calcium signaling pathway was significantly affected by the combined ligand stimulation. Some type of role for calcium influence on iNOS activity and expression has been previously documented [32]. Four proteins directly influenced by calcium display differential phosphorylation due to the combination of CpG and poly I:C: CaM, CaMK2, Pyk2(Fak2) and isoforms of PKC. Inhibitor results showed that the activities of calcium-influenced signaling proteins CaM and CaMK2 were required to induce a NO response (Fig. 3). It is possible that this calcium-based response was the source of the increased NO release following stimulation with CpG and poly I:C. Peptide array results at 1 h post-poly I:C stimulation showed significant differential phosphorylation of CaMK2 isoforms, which indicates that poly I:C can influence calcium related signaling. Though there are no clear results indicating CpG alone was influencing calcium, CaM and CaMK2 were necessary for CpG-induced NO release, this may be due to CaMK2's regulatory role on iNOS. Inhibition of CaMK2 significantly reduced cellular NO production [33]. CaMK2 directly interacts with iNOS and is able to regulate iNOS activity [33]. It has been previously shown that increased calcium resulting in increased activation of CaMK2 resulted in activation of iNOS and increased NO levels [34]. Some type of dependence on calcium for the induction of NO release was further confirmed by the use of the calcium chelator BAPTA. The addition of either 50 μM or 100 μM BAPTA completely eliminated the NO response of monocytes stimulated with CpG or CpG and poly I:C in combination (Fig. 6). A role for calcium in the expression and activity of iNOS may seem controversial as iNOS activity is often considered calcium-independent [35]. However, calcium-related proteins were still required for activity and studies have shown a dependence on CaMK2, CaM and calcium in iNOS expression and activity [34,36,37]. The CaMK2 regulatory role may also provide an evidence for why that with poly I:C alone, no NO release is observed, because at 1 h post stimulation, CaMK2 T286 had undergone a deactivating dephosphorylation at residue T286 (fold change = −1.15, p-value = 0.005) (Supplementary Table 1). At 1 h post stimulation with the combination of CpG and poly I:C, CaMK2 T286 showed a phosphorylation fold change of 1.14 and p-value of 0.029 (Supplementary Table 1).

There are signal transduction pathways which lead from calcium-related proteins to the activation of transcription factors which induce iNOS expression, again indicating that iNOS cannot be considered truly independent of calcium's influence. The combination of CpG and poly I:C induced a stronger calcium signaling response and a larger NO release. This was clearly observed in the results shown in Table 3 and Fig. 2. These effects may have been due to the combined activation of NFκB by both CpG and poly I:C. Both ligands have been shown to activate NFκB [38,39]. NFκB is known to induce an inflammatory response which leads to the release of NO [36]. At all concentrations of NFκB inhibitor, CpG-induced NO release was eliminated; and at the higher concentrations of NFκB inhibitor, synergistic NO release was also eliminated (Fig. 3). Stimulation with CpG at 1 h showed increased phosphorylation of IκB-alpha at S36 (1.59, p-value = 0.036), an inhibitor of NFκB. IκB-alpha phosphorylation results in a separation of IκB and NFκB resulting in NFκB activity [40]. The peptide array data does not show NFκB to be significantly phosphorylated following stimulation with the TLR ligands, but this may be a sensitivity issue, since NFκB phosphorylation is just over the p-value significance threshold of 0.05 in poly I:C and combination stimulated cells (Supplementary Table 1). NFκB is clearly required for NO release as shown in Fig. 3.

CREB is another transcription factor that activates iNOS and induces a NO release [41]. The activation of both NFκB and CREB may have been the trigger for the synergistic NO release observed by the combined ligand stimulation. A synergistic iNOS response with the activation of NFκB and CREB has been reported [41]. It has been known for many years that CREB, in addition to its well- known response to cAMP, can be regulated by calcium and CaM-dependent kinases [42]. The transcription factor CREB was significantly phosphorylated with the combination of ligands at S119 (fold change = 1.35, p-value = 0.025) (Supplementary Table 1), an activating phosphorylation (equivalent to rat CREB S133) [43]. This phosphorylation was not observed with poly I:C or CpG alone. CREB forms a heterodimer with CCAAT/enhancer binding protein (C/EBP) when both are phosphorylated and leads to a synergistic increase in iNOS when cells are treated with LPS and another cytokine [41], a synergistic iNOS action has also been reported between NFκB and C/EBP [44]. With the combination of CpG and poly I:C, the peptide array results showed that CREB is significantly phosphorylated while C/EBP displays increased phosphorylation just on the threshold of significance (fold change = 1.23, p-value = 0.053). A similar CREB–NF-kB-induced synergistic activation of iNOS may be occurring here. These two transcription factors have been shown elsewhere to work in tandem to activate cellular responses; this has been referred to as the CREB–NFκB axis [45]. Fig. 7 shows possible signaling pathways activating CREB and NFκB incorporating signaling proteins shown in this study to be required for NO release.

A classic TLR pathway involves NFκB, PI3K and AKT (KEGG: ko04620). Inhibitor results showed that AKT was required for NO release in all cases (Fig. 3), while the inhibition of PI3K had a lesser effect on NO release (Fig. 5). AKT indirectly influences CREB (KEGG: hsa04151) and NFκB (KEGG: hsa04620). There are several other pathway alternatives in the standard TLR pathway that interact with CREB and NFκB. PI3K activates phospholipase C gamma (PLCγ) (KEGG: hsa04664 + hsa04666) causing calcium release activating CaMK2 (KEGG: hsa04020). PI3K activates Ras-related C3 botulinum toxin substrate 1 (RAC1) which can

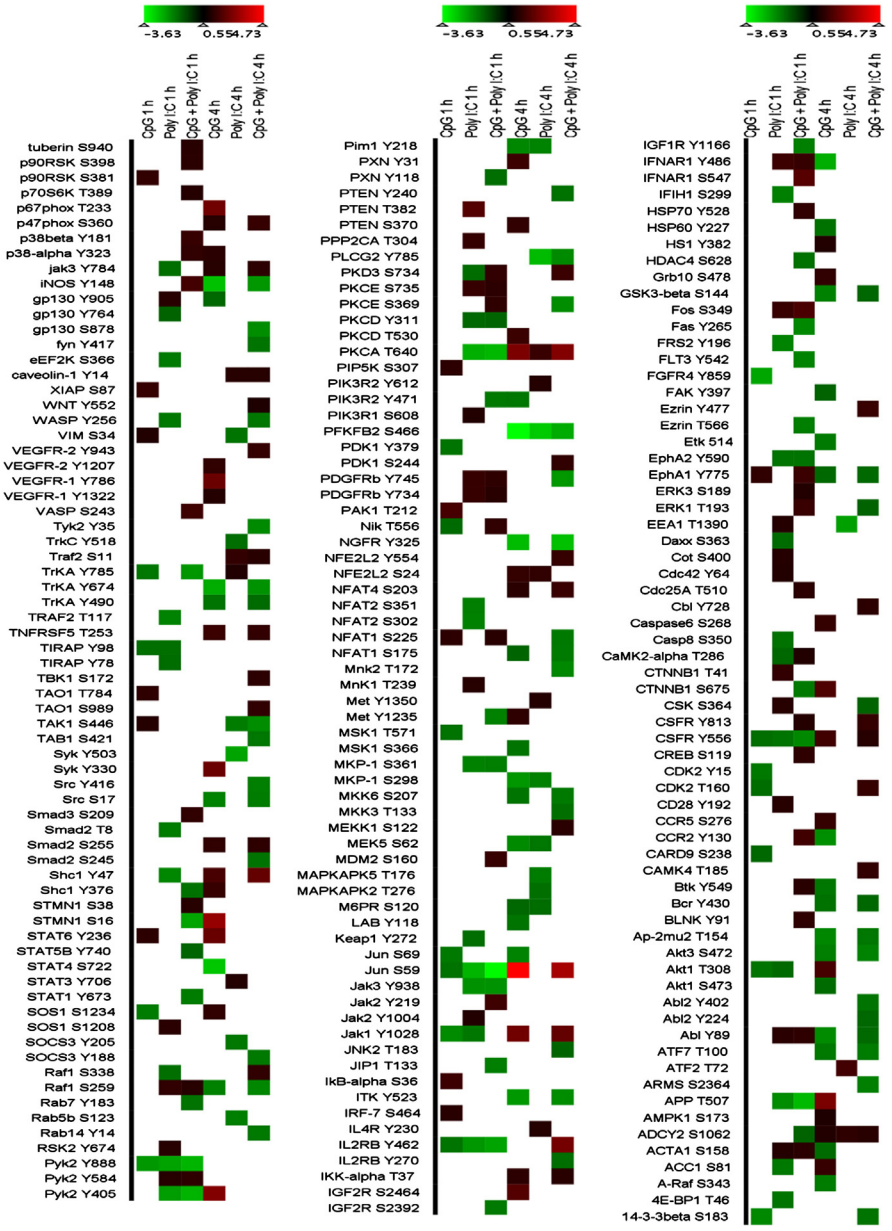

Fig. 7. Secondary signaling pathways leading to iNOS. Signaling diagram of potential secondary synergistic signaling induced by the combination of CpG and poly I:C. All proteins shown, except for RAC, MK2, GSK3, were determined to be involved in TLR synergy by either peptide array or inhibitor assays.

activate NFκB (KEGG: hsa05212). In addition, B cell linker protein (BLNK) and Bruton tyrosine kinase (BTK) are known to activate PLCγ (KEGG: hsa04064), and both of these proteins were shown to be phosphorylated in cells stimulated with CpG and poly I:C (Table 1). The pathway involving apoptosis signal-regulating kinase 1 (ASK1), mitogen-activated protein kinase kinase 3 (MKK3), mitogen-activated protein kinase kinase 6 (MKK6), p38 MAPK, and mitogen-activated protein kinase-activated protein kinase 2 (MK2) activates CREB (hsa04010). Calcium activates protein kinase C (PKC) and the peptide array results showed several isoforms which are phosphorylated by the combination of CpG and poly I:C (Table 1). PKC activates the MAPK signaling cascade via Rat sarcoma (RAS) and rapidly accelerated fibrosarcoma (RAF), ultimately leading to ERK's activation of ribosomal S6 kinase 1 (RSK1 also referred to as p90RSK), which activates CREB (KEGG: hsa04720). PKCδ Y311 is one of the most significantly altered phosphorylation events induced by the CpG and poly I:C combination (Supplementary Table 1). When phosphorylated at Y311, PKCδ stability is altered and the protein undergoes degradation [46]. The peptide array results show that PKCδ is significantly dephosphorylated relative to control (-1.14, p-value $= 0.009$), indicating the protein is stable. One of PKCδ's activities is to stabilize iNOS mRNA enhancing transcription of the enzyme [47]. Many of the signaling intermediates described above are involved in cancer-related pathways. This may explain why a number of cancer pathways were generated by the pathway analysis (Table 2).

In the absence of an inhibitor, as was the case with the peptide array assays, an "all of the above" signaling cascade may be induced by the combination of CpG and poly I:C. Both the classical TLR signaling pathways and the calcium-related secondary pathways (Fig. 7) may be activated. This activation can be observed in the peptide array data for the ligand combination 1 h post-stimulation. This data showed differential phosphorylation of peptides representing proteins discussed here: BLNK, BTK, PKC, RAF, ERK, Jak2, Jak3, p38 MAPK, STAT, PIK3R2, CaMK2, AP-1 (Fos subunit), CREB, iNOS, p90RSK (RSK1) and PKC (Supplementary Table 1). This combined signaling, while at the same time producing signaling events unique to the combined stimulation, could lead to NO

production and release greater than either of the ligands alone. Since many microbes contain within them ligands which can stimulate more than one TLR, this synergistic influence on cellular signaling may be an important consideration for the study of immune response. Novel pathways that are activated by a combination of TLR ligands may add to what we consider to be the canonical TLR signaling pathways.

In conclusion, we have shown that unique signaling is generated by the combination of the TLR ligands CpG and poly I:C in chicken monocytes. This was shown by comparing the phosphorylation changes induced by CpG alone, poly I:C alone and the combination. Unique phosphorylation events were displayed by the combination not seen with the individual ligands. These phosphorylation events included those involving the calcium signaling pathway. One of the immunological results of this ligand combination is the synergistic increase in NO release. Inhibitor assays showed that a number of signaling proteins were required for CpG induced NO release but not ligand combination induced NO release. These proteins included AP-1, TAK1, TBK1 and Jak2/STAT1,3. This result again confirms the unique signaling events induced by the ligand combination. Following CpG and ligand combination stimulation the inhibitor assays indicated a requirement for the calcium regulated proteins CaM and CaMK2. Calcium chelator assays showed an elimination of NO response when calcium was chelated. These results confirmed the requirement for calcium in the TLR induced NO release. Based on our peptide array data we proposed a number signaling mechanisms that would explain the TLR synergistic effects observed, leading to the activation of NFκB and CREB and expression of iNOS.

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